A new generation of biocides for control of crustacea in fish farms.

Peter K. J. Robertson[1*], Kenny Black[2], Morgan Adams[1], Kate Willis[2], Fraser Buchan[1], Heather Muir[2], Linda Lawton[1] and Cathy McCullagh[1]

[1]Centre for Research in Energy and the Environment, The Robert Gordon University, Schoolhill, Aberdeen, AB10 1FR, UK.

[2]Scottish Association for Marine Science, Dunstaffnage Marine Laboratory, Oban, Argyll, Scotland, PA37 1QA

Corresponding author[*].

Peter K.J. Robertson

Centre for Research in Energy and the Environment

The Robert Gordon University

Schoolhill

Aberdeen

AB10 1FR

UK.

Telephone: +44 1224 263750

Fax: +44 1224 263757.

E-mail address: peter.robertson@rgu.ac.uk

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Abstract

Farming of Salmon has become a significant industry in many countries over the past two decades. A major challenge facing this sector is infestation of the salmon by sea lice. The main way of treating salmon for such infestations is the use of medicines such as organophosphates, pyrethrins, hydrogen peroxide or benzoylphenyl ureas. The use of these medicines in fish farms is, however, highly regulated due to concerns about contamination of the wider marine environment. In this paper we report the use of photochemically active biocides for the treatment of a marine copepod, which is a model of parasitic sea lice. Photochemical activation and subsequent photodegradation of PDAs may represent a controllable and environmentally benign option for control of these parasites or other pest organisms in aquaculture.

1. Introduction

The culture of Atlantic salmon (*Salmo salar*) has become a significant industry in countries from both hemispheres over the past 20 years, but in several countries environmental concerns have become significant constraints to further development [1]. The efficiency of the industry has improved with increasing production levels, and husbandry and management techniques continue to advance. Further expansion of the industry in Europe, Canada and Chile, however, is still threatened by the proliferation of ectoparasitic sea lice. Sea lice belonging to the genera *Lepeophtheirus* and *Caligus* (Caligidae: Crustacea) are naturally occurring ectoparasitic copepods of salmonids. Major infestations can weaken the salmon resulting in the development of secondary infections, the transmission of microbial pathogens, and higher mortality rates. In addition, the market value of the fish may be reduced due to unsightly lesions [2]. Sea lice can be transmitted between farmed and wild populations of both salmon and sea trout (*Salmo truta*) [3], thus there are strong pressures from conservationists and regulators to keep lice numbers on farmed fish to a minimum.

Economically viable salmon farming would not be possible without controlling parasitic infestations using medicines [4], which are administered to caged salmon as bath immersion treatments or in salmon feed. A range of compounds with differing modes of action have been, or are still being used for the control of sea lice on commercial salmon farms worldwide. These included two organophosphates (dichlorvos, Salmosan); three natural

pyrethrin/pyrethroid compounds (pyrethrum, Excis, deltamethrin); one oxidizing agent (hydrogen peroxide); three avermectins (ivermectin, emamectin, doramectin) and two benzoylphenyl ureas (teflubenzuron, diflubenzuron). Bath immersion treatments are administered to lice infected salmon by surrounding a cage with a tarpaulin, which is removed at the end of the treatment, releasing the solution into the surrounding water where it disperses in the direction of prevailing current flow. In-feed treatments are administered to salmon incorporated into feed, and may enter the marine environment either directly from waste feed, indirectly via faeces during the treatment period or by egestion post-treatment. Because of the non-specific toxicity and potential environmental impacts of the currently available sea lice treatment medicines, they are highly regulated to reduce the likelihood of adverse affects on the surrounding marine environment. Consequently, commercial salmon farm production may be limited by the amount and type of treatment agent that farms are licensed to use during a production cycle. Therefore, a sea lice treatment agent that is highly specific but looses its toxicity following treatment so that it does not adversely affect the wider marine environment when it is released would be beneficial both for the industry and the environment.

Photodynamic therapy employs the combination of light and a drug to bring about a cyto-toxic or modifying effect to cancerous or otherwise unwanted tissue [5, 6]. A photosensitiser which exhibits negligible toxicity in the dark is introduced into the body and accumulates preferentially in rapidly dividing

cells. When the photosensitiser attains an appropriate ratio of accumulation in diseased versus healthy tissue, a carefully regulated light dose is applied to the diseased tissue. The light activates the photosensitiser and elicits its toxic action [6, 7]. PDT is dependent on the presence of molecular oxygen [7]. This suggests that singlet oxygen generated by the photosensitisation of molecular triplet oxygen is the principal toxic species produced during PDT, although the extent to which this species is responsible for the photodynamic effect is under debate [8, 9]. Nonetheless, the generation of singlet oxygen is extremely crucial to the success of PDT, and one of the first tests performed on potential PDT compounds is an investigation of their ability to produce singlet oxygen [9].

Methylene Blue (MB) and Nuclear Fast Red (NFR) are two known photosensitisers. MB is a phenothiazinium dye whose efficacy against the methicillin-resistant *Staphylococcus aureus* (MRSA), a major cause of nosocomial infection [10], has been investigated. The uptake of dyes and stains by bacteria has long been used in their detection, and several such dyes are inherently bactericidal [11]. Studies on the use of phenothiazines in the photodynamic therapy of cancer have concentrated predominantly on MB and its demethylated analogues azure C, thionine and toluidine blue [12]. MB exhibits phototoxicity toward a variety of tumour cell lines *in vitro* [13-18]. NFR is an anthraquinone dye, and a number of anthraquinones, both synthetic and naturally occurring, have been screened for their anti-tumour activity in a variety of animal test systems [19-21]. Many such

anthraquinone derivatives possess the ability to mediate single electron transfer to molecular oxygen to form a superoxide anion radical and to generate reactive oxygen species (ROS) when stimulated by visible light [22, 23].

Photosensitive compounds may offer an environmentally friendly alternative to the compounds currently used to control sea lice on salmon farms. We have previously reported the use of methylene blue (MB) and nuclear fast red (NFR) as biocides in the treatment of algae and cyanobacteria [24-26]. This paper presents the results of an assessment of the suitability of these two photosensitisers, including bioassays with the copepod *Acartia clausi*, to assess their toxicity following light activation.

The marine copepod *Acartia clausi* was used as a model organism in place of sea lice to assess the toxicity of light activated MB and NFR. *A. clausi* belongs to the same Subclass (Copepoda) as sea lice, but unlike the target organism, *Lepeophtheirus Salmonis*, is easily cultured in the laboratory, and has also been used previously to assess the toxicity of sea lice treatment medicines [27, 28]. Given the close relationship and similarity in life cycles of the two species, *A. clausi* was considered to be an appropriate substitute in this preliminary assessment of the potential use of photoactivated biocides to control sea lice.

2. Materials and Methods

2.1 Materials

Methylene Blue (85%) was purchased from Fisher Scientific, Nuclear Fast Red was purchased from Sigma and 1,3-Diphenylisobenzofuran (97%) was purchased from Aldrich. The photosensitisers were prepared in 0.2 μM GF/C filtered sea water. 1,3-Diphenylisobenzofuran was prepared in methanol.

Acartia clausi were collected using vertical hauls of a 120 μm zooplankton net from sea lochs on the west coast of Scotland close to Dunstaffnage Marine Laboratory, Oban. On return to the laboratory, adult copepods were sorted and identified according to Sars [29]. They were transferred into culture vessels (2.5 L plastic buckets) containing 0.2 μm GF/C filtered sea water and continuously aerated. Cultures were maintained at 13 ± 1^{o}C in a temperature-controlled room under dim light, with a photoperiod of 14 h light and 10 h dark. Copepods were fed daily to excess with *Rhinomonas reticulata* var. *reticulata* (995/2 Culture Collection of Marine Algae and Protozoa, CCAP). Algal cultures were maintained in Walne's medium (*30*) in 10 L closed carboys at 20 ± 1^{o}C under continuous fluorescent light. Copepod culture vessels were cleaned and the media changed twice weekly. To ensure a regular supply of animals of known age for use in toxicity tests, new cultures were started every 2 to 4 days with eggs and nauplii separated from adult cultures.

2.2 Photochemical reactions

Aqueous solutions of Methylene Blue (MB) (10 μM) and Nuclear Fast Red (NFR) (10μM) were prepared from stock solutions. A solution of each dye (50 cm^3) was exposed to illumination from a 500 W tungsten halogen lamp in open Pyrex flasks (100 cm^3) for a period of 60 or 120 minutes. Samples (3 cm^3) were taken at either 5 or 10 minute intervals and the fluorescence of the samples monitored. Each experiment was repeated 3 times and dark controls were carried out simultaneously. Solutions containing a mixture of MB:NFR (10:90, 25:75, 40:60, 50:50) was exposed to illumination from a 500 W tungsten halogen lamp in open Pyrex flasks for 60 minutes. The solutions were sampled at the time intervals described previously. The irradiated samples were analysed using a luminescence spectrometer (Perkin-Elmer LS B50). The excitation and emission wavelengths for fluorescence monitoring of MB and NFR were 667 nm:691 nm, and 545 nm:595 nm, respectively.

2.3 Singlet oxygen determinations

The yields of singlet oxygen for MB and NFR were determined using the 1,3-Diphenylisobenzofuran (1,3-DPBF) bleaching method [31,32]. 1,3-DPBF is an established singlet oxygen scavenger and through the decrease in 1,3-DPBF absorption, monitored spectroscopically, the efficiency of the dyes at generating singlet oxygen could be subsequently monitored. Solutions of the photosensitisers (MB and NFR) and 1,3-Diphenylisobenzofuran (1,3-DPBF) were illuminated under visible light. The rate at which the furan was

consumed was followed spectrophotometrically by observing the decrease of an absorption band at 410nm as a function of irradiation time. In order to improve the accuracy in comparing singlet oxygen quantum efficiencies each photosensitiser was evaluated under similar experimental conditions. To ensure that an equal number of photons were absorbed per unit time in all experiments, the concentration of each photosensitiser was adjusted to give a maximum absorption at their respective absorption maxima. The concentration of 1,3-DPBF utilised was that which achieved a maximum absorption at 410nm. A solution of MB containing 1,3-DPBF was exposed to irradiation by visible light, samples were taken ever 60 seconds and the absorbance of 1,3-DPBF monitored for a period of 10 minutes or until the readings became negligible. This procedure was repeated for NFR.

2.4 Copepod toxicity tests

The toxicity of each PDA and a "cocktail" of MB/NFR (25:75) to the marine copepod *A. clausi* were investigated in 24 h static tests. Adult copepods were exposed to five concentrations (10^{-8}M to 10^{-4}M) and a control, with three replicates of 10 animals per concentration. Animals were transferred into test solutions using disposable Pasteur pipettes in a minimum of sea water to avoid dilution. Exposure vessels were 50 cm^3 borosilicate glass beakers containing 40 cm^3 of test solution. Tests were undertaken in a temperature-controlled room (13 ± 1^{o}C) under ambient light. The highest concentration in the toxicity tests was that used to determine the absorption spectra of each PDA, i.e. 10^{-4} M. Mortality was assessed after 24 h under a stereomicroscope.

Mortality was defined as a lack of movement when gently prodded with a blunt needle. Tests were considered successful if control survival was greater than 90%.

The toxicity of the PDAs to adult copepods following light-activation was assessed as described above, but with the inclusion of a 1 h exposure period to white light to activate the PDAs. Adult copepods were exposed to the same range of PDA concentrations as above under ambient light. After an initial exposure period of 1 h the test chambers were placed under white light for 1 h to activate the PDA. The test chambers were then placed back under ambient light, and after 24 h, copepod mortality was assessed as above.

3. Results and discussion

3.1 Effect of illumination on the lifetime of photosensitisers

To determine the most effective concentrations of MB and NFR for use in the toxicity experiments with *A. clausi*, a range of concentrations of both photosensitisers were screened. Their response to irradiation for 120 minutes from a visible light source was monitored. MB and NFR were studied from 0.01 μM to 100 μM, these concentrations had favourable absorption and emission spectra without any quenching.

Within experimental error, the fluorescence of MB (10 μM) did not decrease after 60 minutes illumination when exposed to visible light (Figure 1a). In contrast, Nuclear Fast Red (10 μM) showed efficient breakdown in visible light indicated by a decrease in fluorescence (and a colour change) which was proportional to irradiation time (Figure 1b). The decrease in fluorescence of NFR when irradiated in solution with MB is shown in Figure 1c. Various combinations of MB:NFR (10:90; 25:75; 40:60; 50:50) were investigated to determine the optimum ratio for NFR breakdown. The presence of MB increases the rate of breakdown of NFR in each ratio. The fluorescence of MB does not decrease over the course of the irradiation time suggestive that it is not affected by the addition of NFR. The optimum ratio for the photo-destruction of NFR was 25:75 where the rate of breakdown of NFR increased when compared to fig. 1b where NFR was irradiated alone. The 25:75, MB:NFR ratio was repeated and sampled at 60 second intervals, fig. 2. After 23 minutes irradiation the fluorescence of NFR was negligible. Fig. 1b

illustrates that after 23 minutes irradition of NFR alone the fluoresence had only decreased by 27% of the initial reading. Combining NFR with MB and irradiation did not cause the fluorescence of MB to decrease.

3.2 Determination of singlet oxygen production

An important factor in the use of photosensitisers is their ability to produce singlet oxygen. This is the toxic element and the production of singlet oxygen needs to be determined in order to determine whether the photosensitiers proceed via a type 1 or type 2 reaction. Upon illumination with visible light the oxidation of cells proceeds via two competitive mechanisms. The type I mechanism produces a radical intermediate by direct interaction of the light excited photosensitiser (MB*) with the substrate via electron transfer. The type II mechanism involves energy transfer from the photo-excited state of the photosensitiser to oxygen with the production of singlet oxygen [33]. Singlet oxygen is the lowest electronically excited state and a mutagenic form of molecular oxygen [34].

Fig. 3a illustrates the decrease in absorption of 1, 3-DPBF under dark and light conditions in the presence of MB. There was an immediate and rapid decrease in the absorbance of 1, 3-DBPF after 60 seconds illumination indicating a high singlet oxygen yield. However, in the dark, there was no breakdown of the 1, 3-DPBF, which makes it evident that light activation of MB is required to produce singlet oxygen, and consequently have a toxic effect on the target organisms.

Fig. 3b illustrates the decrease in absorption of 1,3-DBPF under dark and light conditions in the presence of NFR. Irradiation of the sample effects a decrease in the absorption of 1,3-DPBF as a result of the production of singlet oxygen from NFR. It is a relatively slower process than that recorded for MB, figure 3b. The conclusion to be drawn is that MB is a better producer of singlet oxygen than NFR under these conditions. Under dark conditions the absorbance of 1,3-DBPF remains at the same level confirming that NFR requires visible light irradiation for activation.

Fig. 3c illustrates the singlet oxygen production ability of the 25:75 MB:NFR mixture. The absorbance of 1,3-DPBF decreases with irradiation similarly to the previous cases. However the amount of singlet oxygen produced is greater than in the case of NFR alone, and less than in the MB alone situation. Correlating the singlet oxygen results to the photochemical reactions, it is evident that the increased activity observed during irradiation of a solution of MB:NFR, towards the breakdown of NFR, results from an increased production of singlet oxygen. The singlet oxygen results also demonstrate that MB is a superior producer of singlet oxygen than NFR. Structurally NFR is more susceptible to alteration via reaction with singlet oxygen and this is observed with a decrease in the fluorescence of NFR.

3.3 PDA toxicity to Acartia clausi

Copepod mortality rates following 24 h exposure to MB, NFR and the "cocktail" under ambient light and darkness were compared with the results

of tests that included a light activation period of 1 h (Fig. 4a-c). Copepod mortality following exposure to MB at concentrations of 10^{-6} M and 10^{-5} M was considerably higher (90% and 95% respectively) when the exposure period included light activation, than under ambient light or darkness (Fig. 4a). At the highest concentration of 10^{-4} M, mortality rates were similar for all treatment conditions.

Mortality rates were low (<15%) in all NFR concentrations under all treatment conditions, and light activation did not increase the toxicity of NFR to adult copepods (Fig. 4b). This may be because the NFR flocculated out of solution in the higher concentrations and was unavailable for uptake by the copepods, or because of lower singlet oxygen production (Fig. 3b)

Toxicity of the PDA "cocktail" (NFR/MB) to adult *A. clausi* increased greatly when the exposure duration included a 1 h light activation period (Fig. 4c). At a concentration of 10^{-6} M, copepod mortality was approximately 55% with a 1 h light activation period. At the higher concentrations of 10^{-5} and 10^{-4} M, 95% and 100% mortality were observed respectively following light activation. Exposure under ambient light and dark conditions only resulted in mortality rates of less than 20% and 50% respectively, at the two highest concentrations. Mortality was higher under darkness than under ambient light in the two higher "cocktail" concentrations.

For comparative purposes, some toxicity data are available for the active ingredients in 2 anti-sea lice medicines - emamectin benzoate [27] and cypermethrin [28] - in acute tests with the same model organism adult *A. clausi*. The 48h EC50s were 2.9 x 10^{-10} M and 6.4 x 10^{-9} M for emamectin benzoate and cypermethrin respectively. In the present work, toxicity was determined over a shorter period (24h) but even so the concentrations were much higher indicating that the PDAs are less toxic than the current generation of active-ingredients. Whether this is a beneficial attribute requires to be established in further testing (section 3.4).

3.4 Photosensitisers for Sealice control

Acartia clausi was used as a model for copepod sea lice in order to test the concept of the use of photosensitisers for the control of sea lice. As it is not possible to conduct experiments on post-larval phases of sea lice except on infected fish, the next step would be to test the relative toxicity of these photosensitisers to sea lice and infected salmonid hosts. This would establish whether a therapeutic window exists i.e. whether there is a sufficient difference in toxicity towards the parasites and their hosts to allow effective treatment. The sensitivity of the therapeutic window to other environmental parameters would also require to be determined – for example, hydrogen peroxide has been used as a sea lice treatment but has limited use in late summer as its therapeutic window diminishes to zero at around 14°C water temperature [35]. In the host fish, not only acute toxicity but also chronic effects, such as colour and taint, would require study. Assuming such studies

proved positive, and the photosensitisers were shown to be relatively safe in terms of fish health and residues, a potential product developer would need to research formulation and application technologies and provide commercial-scale trial data to provide the information required for the Marketing Authorisation process. Like hydrogen peroxide, the photosensitisers examined here are relatively cheap generic products, and the protect-able intellectual property would likely reside in the specific formulation and treatment process developed.

4. Conclusions

The use of photochemically active biocides for the treatment of a marine copepod, which is a model of parasitic sea lice has been demonstrated. The process was effective when methylene blue and a mixture of methylene blue and nuclear fast red reagents were irradiated with visible light. Nuclear fast red was however less effective with little evidence of copepod mortality achieved even after 60 minutes irradiation. The results of this study would suggest that photochemical activation and subsequent photodegradation of PDAs may represent a controllable and environmentally benign option for control of these parasites or other pest organisms in aquaculture. However, there are significant challenges that must be overcome before any new product can be brought into the market including: efficacy, fish safety, environmental safety and the development of a patentable application. The growing resistance to the few existing products for sea lice treatment [36]

may incentivise a potential developer to undertake the first stages of this process.

Acknowledgements

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Abbreviations:

MB: Methylene Blue

NFR: Nuclear Fast Red

1,3-DPBF: 1,3-Diphenylisobenzofuran

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Captions for Figures

Fig. 1: Plots of: (a)fluorescence of MB versus time with irradiation; (b) fluorescence of NFR versus time with irradiation; (c) fluorescence of NFR versus time with irradiation in combination with MB at different ratios: 90:10; 75:25; 50:50; 40:60.

Fig. 2: Effect of irradiation and MB on the fluorescence of NFR.

Fig. 3: Singlet oxygen production from (a) irradiated MB in presence of 1,3-DPBF; (b) irradiated NFR in presence of 1,3-DPBF; (c) irradiated NFR:MB, 75:25, in presence of 1,3-DPBF.

Fig. 4. Mortality of adult *Acartia clausi* following 24 h exposure to (a) Methylene Blue, (b) Nuclear Fast Red, and (c) a "cocktail" of MB and NFR (25:75) under ambient light, darkness, and when the exposure duration included a light activation period of 1 h.

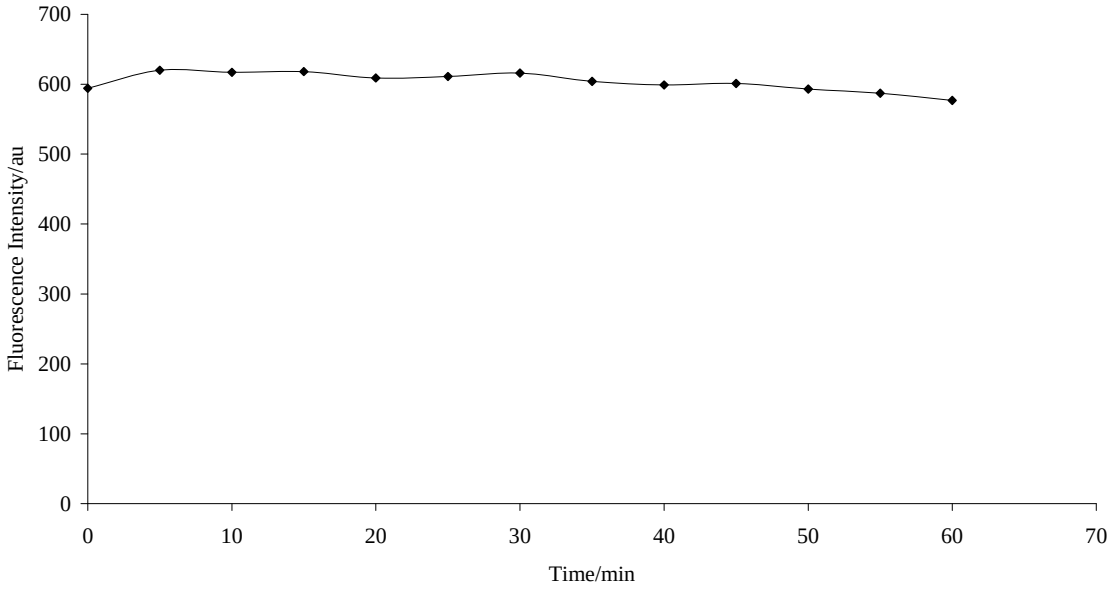
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Fig 1a

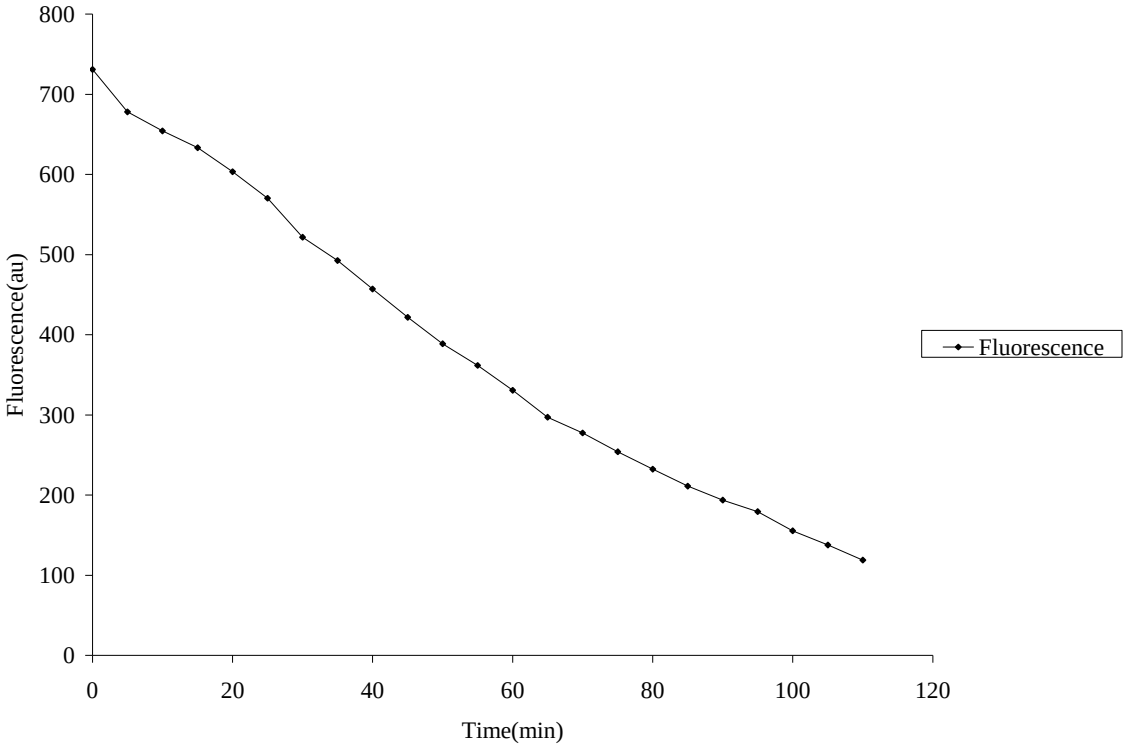
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Fig 1b

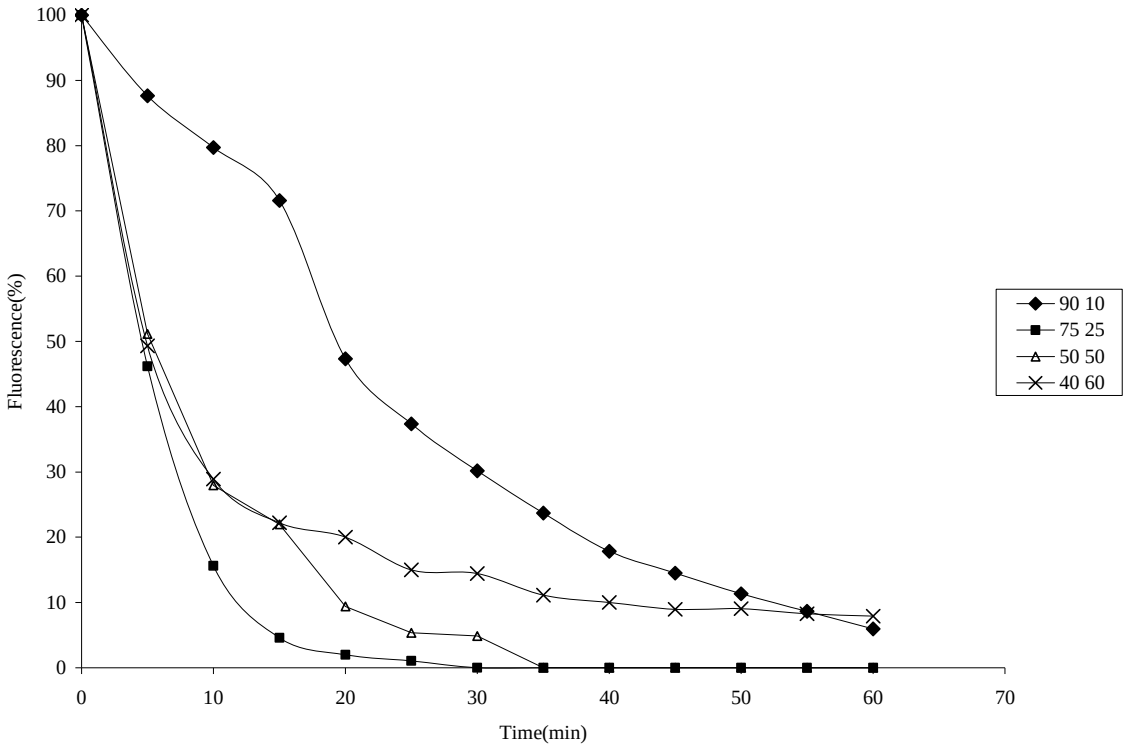
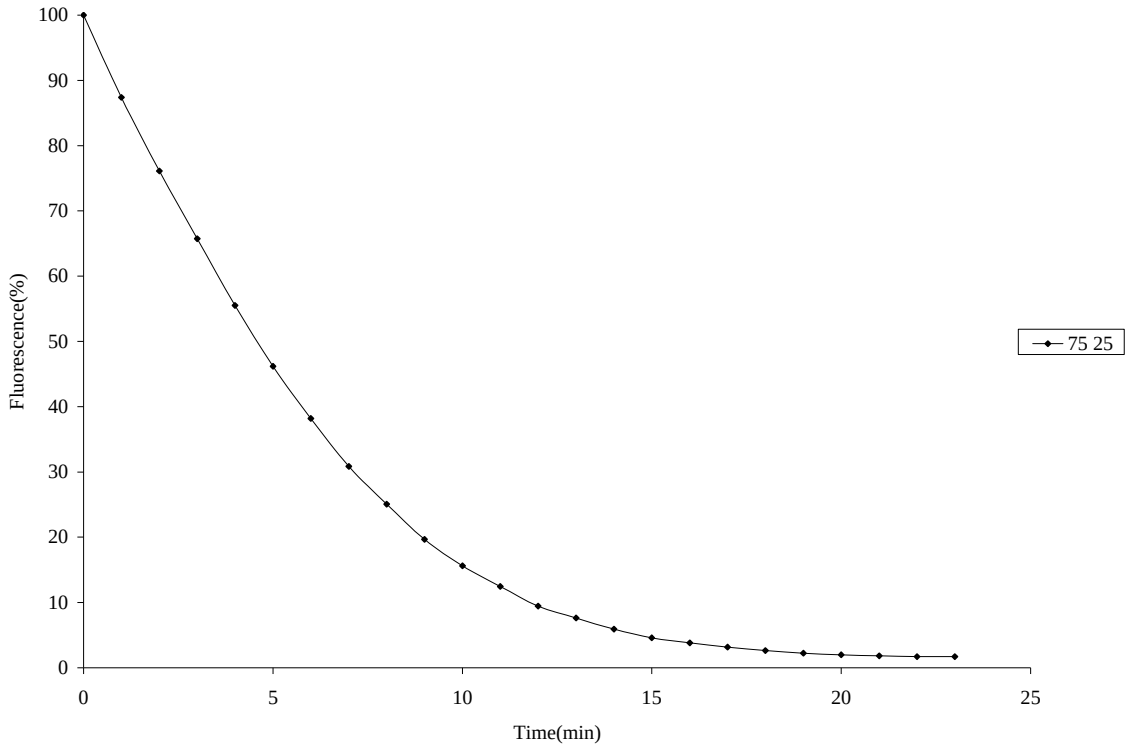


Fig 1c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Fluorescence(%)
0
5
10
15
20
25
Time(min)
75 25

Fig 2

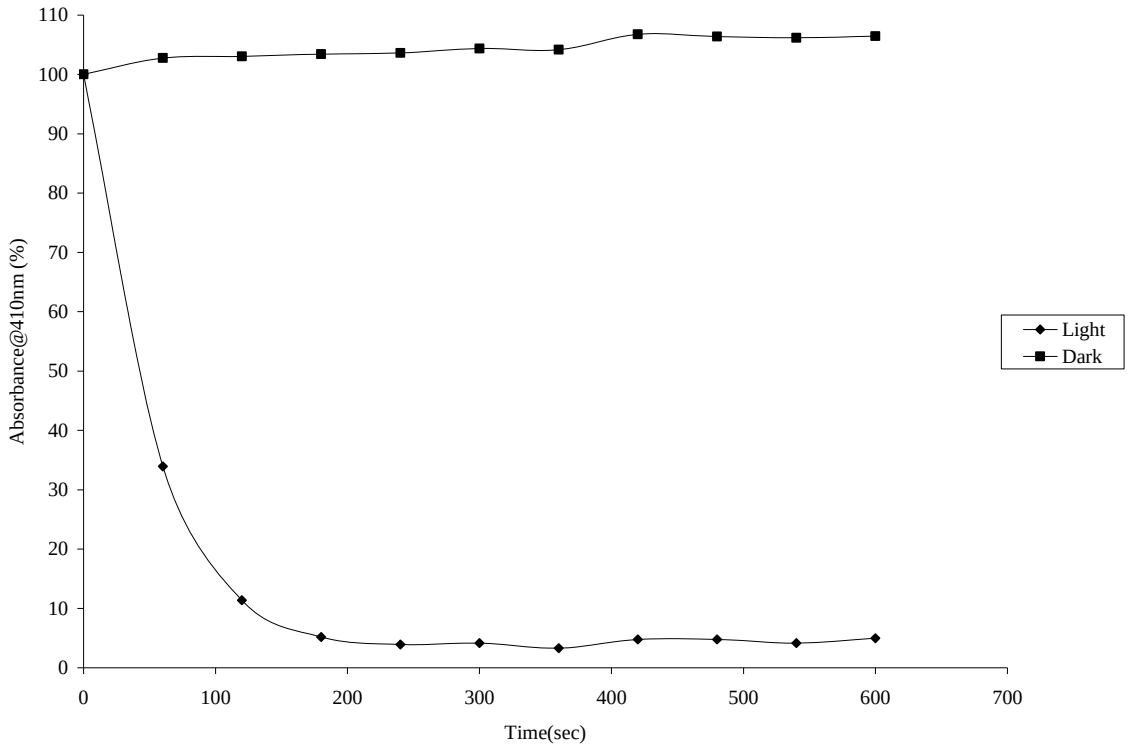
110
100
90
80
70
60
50
40
30
20
10
0
Absorbance@410nm (%)
0
100
200
300
400
500
600
700
Time(sec)
Light
Dark

Fig 3a

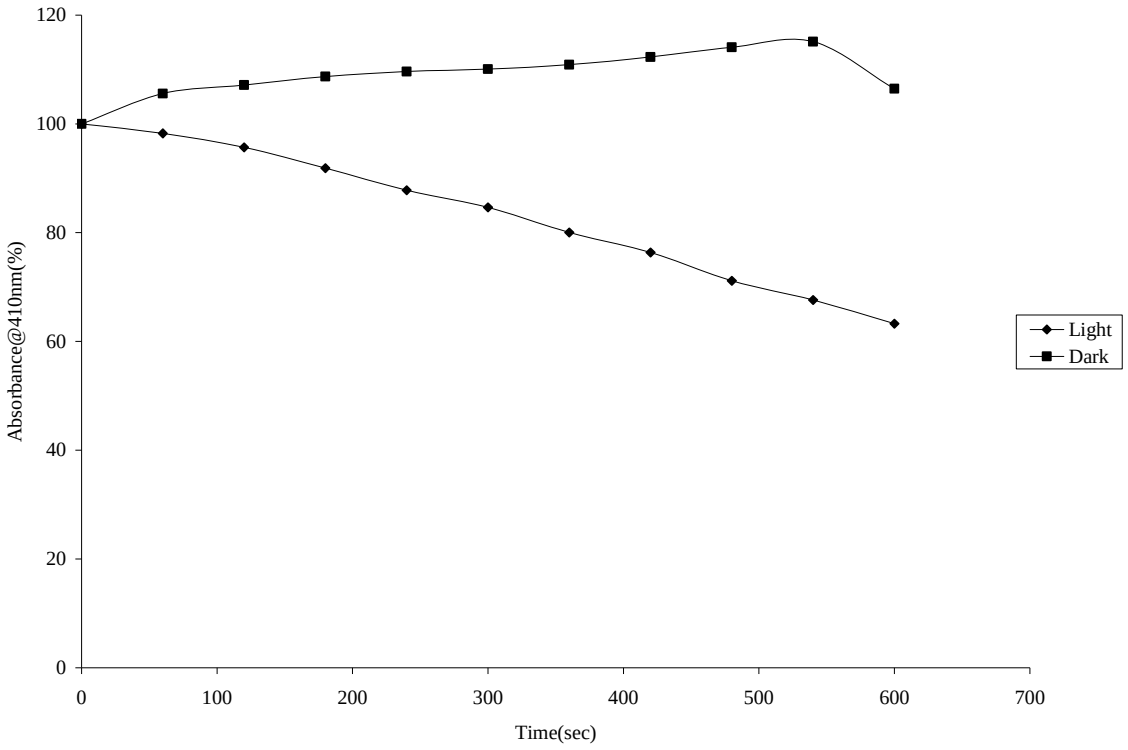
120
100
80
60
40
20
0
Absorbance@410nm(%)
0
100
200
300
400
500
600
700
Time(sec)
Light
Dark

Fig 3b

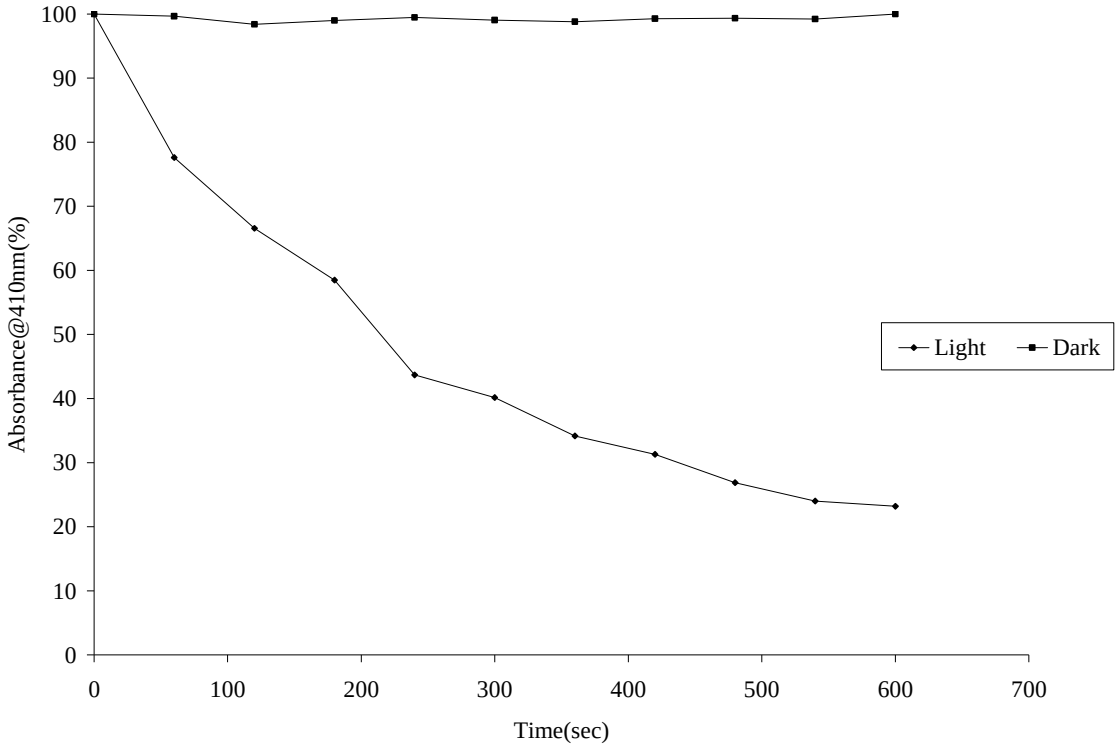
100
90
80
70
60
50
40
30
20
10
0
Absorbance@410nm(%)
0
100
200
300
400
500
600
700
Time(sec)
Light
Dark

Fig 3c

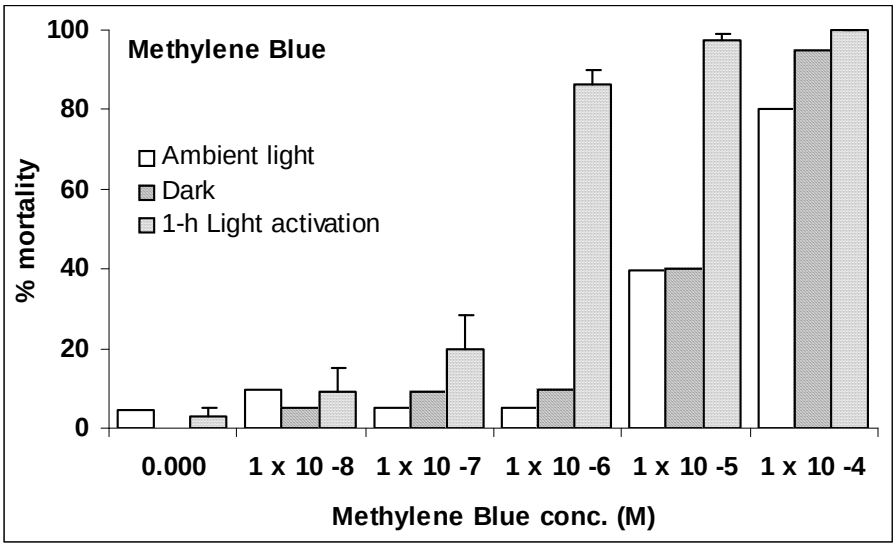
Methylene Blue
Ambient light
Dark
1-h Light activation
% mortality
100
80
60
40
20
0
0.000
1 x 10 -8
1 x 10 -7
1 x 10 -6
1 x 10 -5
1 x 10 -4
Methylene Blue conc. (M)

Fig 4a

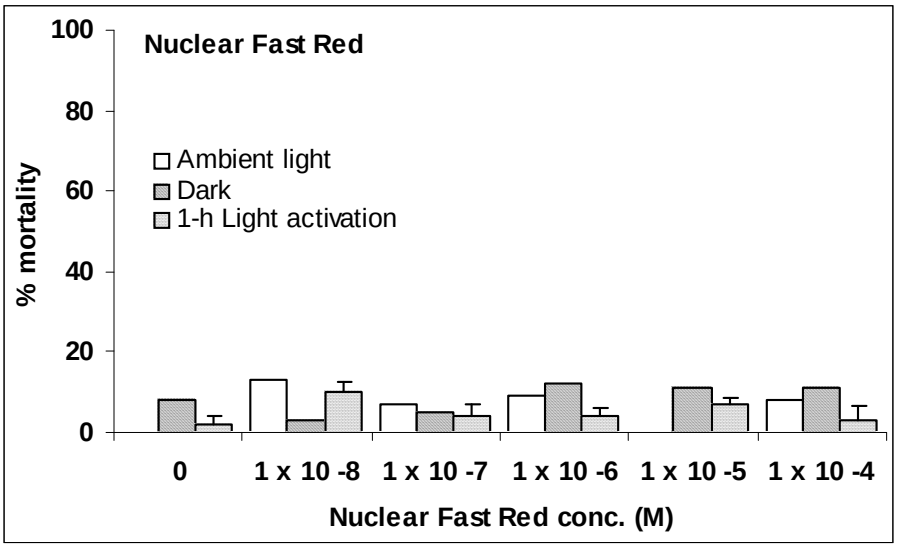
Nuclear Fast Red
100
80
60
40
20
0
% mortality
Ambient light
Dark
1-h Light activation
0
1 x 10 -8
1 x 10 -7
1 x 10 -6
1 x 10 -5
1 x 10 -4
Nuclear Fast Red conc. (M)

Fig 4b

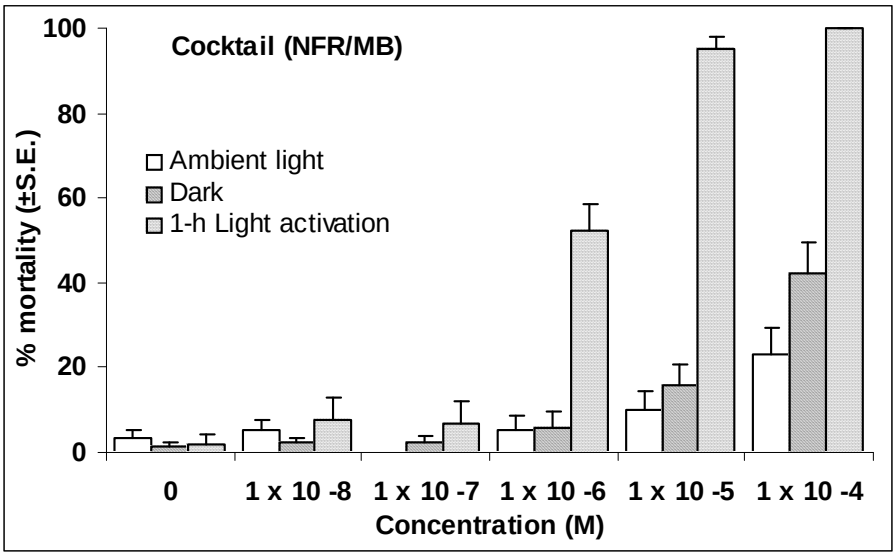
Cocktail (NFR/MB)
Ambient light
Dark
1-h Light activation
% mortality (±S.E.)
100
80
60
40
20
0
0
1 x 10 -8
1 x 10 -7
1 x 10 -6
1 x 10 -5
1 x 10 -4
Concentration (M)

Fig 4c